STATISTICAL OPTIMIZATION OF FERMENTATION PARAMETERS USING PLACKET-BURMAN FOR ENHANCED XYLITOL PRODUCTION BY PICHIA STIPITIS NCIM 3498.

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Abstract

Xylitol a polyol, gaining its importance in food and pharmacological industry due to its low calorific value, anti-cariogenic nature and effective in treating diabetes, anaemia, acute otitis media and osteoporosis. Physico-chemical factors such as temperature, pH, rpm, inoculum size, xylose concentration, co-substrate and nitrogen source play a crucial role on xylitol production. Influence of above said variables on production of xylitol by one factor at a time (OFA T) strategy using Pichia stipitis NCIM 3498 was performed and 30°C, pH5, 200rpm, 48h, 15% inoculum, 7% xylose with glucose as co-substrate and NH₄NO₃ as nitrogen source was found to be optimum for xylitol production. Further, when Plackett-Burman statistical model was used to screen effective medium components, variables like xylose, peptone, magnesium sulphate, ammonium nitrate and di-sodium hydrogen phosphate were found to be highly significant for xylitol production and represented a confidence level of 97%. Validation of xylitol production under optimum conditions has shown maximum xylitol yield concentration and productivity of 52g/l, 0.74g/g and 1.08g/l/h respectively. Xylitol production has increased nearly threefold from 19 g/l to 52g/l using Plackett Burman design.

Key words: xylitol, Anaemia, Osteoporosis, (OFA T), Plackett-Burman.

Introduction

As the consumers are health and weight consciousness, they are inclining towards sugar-free and low-calorie food products due to which the demand for xylitol is increasing (Rao et al., 2016). Xylitol is a low-calorie five-carbon pentitol, became an attractive alternative sweetener for the treatment of diabetes as it is insulin independent Xylitol has numerous applications in the preparation of food, pharmaceutical and cosmetic products (Zhang et al., 2014; Tizazu et al., 2018). It has high heat of dissolution and endothermic solution of heat (34.8 cal g/l), that gives pleasant cool and fresh mint sensation, hence used in the manufacture of oral products like chewing gum, toothpaste, mouthwash (Branco et al., 2011).

Xylitol is anti-cariogenic, it inhibits Streptococcus mutans and avoid acid formation that attack the tooth enamel (Ramesh et al., 2013; Mohamad et al., 2015). Xylitol considerably improves the biomechanical properties of the bones and prevents the reduction in density, calcium and phosphorus mineral content, hence used for the treatment of osteoporosis (Jeevan et al., 2011). Erythrocytic glucose-6-phosphate dehydrogenase deficiency can also be treated with xylitol (J.K. Goli et al., 2012).

Industrial xylitol production is carried out by chemical synthesis which involves toxic catalyst and operated at increased temperature and pressure with expensive steps of purification. Hence microbial production of xylitol is gaining the attention of researchers (Kogie and Ghosalkar 2016; Guirimand et al., 2019). Production of xylitol by microorganisms is an environmentally friendly process as it does not need the use of toxic catalysts nor produces any racemic mixtures and is environmentally safe (Deng et al., 2014). Bacteria, fungi and yeasts like microbes are usually studied for production of xylitol. Especially those belonging to Candida guilliermondii, Debaryomyces hansenii, Candida tropicalis, Candida boidinii, Pachysolen tannophilus and Pichia stipitis are known to be the best xylitol producers (Prakasham et al., 2009; López-Linares et al., 2018). Xylose-assimilating yeasts have xylose reductase (XR) which
reduces D-xylose to xylitol by oxidation of NADPH. Whereas xylitol dehydrogenase (XDH) which cause the oxidation of xylitol to D-xylulose which in presence of xylulose kinase gets converted into D-xylulose 5-phosphate that finally enters the pentose phosphate pathway (Rafiqul et al., 2015).

The current study is to evaluate the ability of Pichia stipitis NCIM 3498 for enhanced xylitol production from pure D-xylose using Plackett Burman statistical design.

Materials and Methods

Microorganism and maintenance

Pichia stipitis NCIM 3498 was procured from NCIM, Pune and was maintained on YEPX (yeast extract-peptone-xylose) agar containing (g/l) yeast extract, 10; peptone, 20; Xylose, 30 and agar, 25; pH, 5.0 and stored at 4°C.

Inoculum preparation

A loopful of culture from agar slant was transferred in 250 ml Erlenmeyer flask that contained 50 ml of medium (g/l): D-xylose 30; yeast extract 3; peptone 5 and were then incubated in an shaker incubator at 150 rpm, 30°C for 24 h. The broth was centrifuged at 10,000 rpm for 10 min and the cell pellet obtained was washed, resuspended in sterile distilled water and further used for fermentation.

Effect of agitation

To find out the agitation effect on growth of organism and xylitol production, fermentation was carried out by incubation of inoculated media at 30°C for 96h by agitating at various rpm such as 100, 150, 200 and 250. Sampling was done after every 24h to estimate the concentration of xylose utilized and xylitol produced.

Effect of temperature

To determine the temperature effect on xylitol production, fermentation was conducted at temperature range of 30°, 35°, 40° and 45°C respectively at 200rpm for 96h. The fermentative broth was collected after every 24h of incubation, centrifuged and the supernatant was estimated for leftover xylose and xylitol concentrations.

Effect of pH

To find out optimum pH on production of xylitol by P. stipitis, the fermentation media is maintained at different pH such as 4, 5, 6 and 7 and left for incubation at 30°C, 200 rpm for 96 h. For every 24h of incubation, samples were collected and estimated for xylose utilized and xylitol produced.

Effect of size of inoculum

To determine the effect of inoculum size on xylitol production, fermentation has been carried out at 30°C, 200 rpm for 96h by varying the inoculum percentage from 5, 10, 15 to 20.

Effect of nitrogen source

The effect of various nitrogen sources on xylitol fermentation was determined by incorporating 1.5% of various nitrogen sources like peptone, urea, ammonium sulphate, ammonium nitrate to the basal media and incubated at 30°C, 200 rpm for 96h.

Effect of co-substrate concentration

The influence of co-substrate on xylitol production was determined by adding 1.5% of different sugars like arabinose, glucose, maltose and galactose to the basal media and incubated at 30°C, 200 rpm for 96 h. The amount of xylose and xylitol in the fermentative broth was analyzed after every 24 h.

Effect of initial xylose on xylitol yield

To evaluate the optimum xylose concentration on xylitol production, fermentation is carried by varying the concentration of xylose from 2% to 8% and incubated at 30°C for 96h at 200 rpm. Samples were estimated for concentration of xylose and xylitol after every 24 h for.

Fermentation conditions

One-factor-at-a-time strategy was followed for initial optimization of physicochemical parameters by using basic media containing g/l xylose 50; yeast extract 1.5; peptone 1.5; MgSO₄·7H₂O, 1; CaCl₂·7H₂O 0.5 and KH₂PO₄ 0.5. However, the composition of medium for Plackett Burman studies was varied according to the design. The media is inoculated with 15% (v/v) inoculum of P. stipitis and incubated at 30°C with 200 rpm of agitation. Samples were collected after every 24h and centrifuged at 10,000g for 10min. The supernatant was filtered with a 0.02 -micron cellulose filter and analyzed for xylitol and residual sugar concentration using High-Performance Liquid Chromatography (HPLC) (Schimadzu). The growth was estimated using UV-VIS spectrophotometer by determining the optical density of the culture at 600 nm (OD600).

Analytical procedure

The concentrations of xylitol and leftover sugars were assessed using HPLC fitted with Repromer Ca column (USP-L19) (9 μm. 300 × 8 mm) (Dr Maisch GmbH, Germany). Water is used as the mobile phase with a flow rate of 0.6 ml/min and at column temperature 75°C using the RI detector.

Experimental design

Plackett-Burman experimental design for the screening different medium components Plackett Burman model is a statistical design used for identification of critical

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parameters during medium optimization. This design evaluates \(N-1\) variables by \(n\) number of experiments. The factors designated \(X1-X12\) represents medium constituents. A total of 12 factors were selected for this study which includes two levels of concentrations for each factor. Every variable is represented at higher concentration (+) and lower concentration (-) i.e. two levels in 20 trials or runs. Each row represents a trial and each column an independent variable. (Table 1). The total number of positive and negative signs per trial is \((K+1)/2\) and \((K-1)/2\), respectively (Table 1). Every column should possess positive and negative signs in equal number. The following equation is used to find out effect of each variable.

\[
E_{xi} = 2\left(\sum M_i^+ - \sum M_i^-\right)/N.
\]

Where \(E_{xi}\) is the effect of concentration of variables tested and \(M_i^+\) and \(M_i^-\) represent the quantity of xylitol produced from the trials. The variable \((X_i)\) measured is available at high and low concentration with number of trials represented by \(N\). The concentration of xylitol produced in g/L, was calculated as the response. All experiments were carried in duplicates and by using t-test the coefficient, the effect and significant level of each variable was calculated.

### Results and Discussion

One factor at a time approach (OFAT) is a simple experimental strategy where the effects of individual factors on production of xylitol can be observed on graphs. Influence of factors like agitation speed, temperature, pH, inoculum percentage, nitrogen source, initial xylose concentration and co-substrate on xylitol production from synthetic media was determined by OFAT as follows.

#### Effect of agitation speed on xylitol production

Oxygen plays a major role in fermentation of xylitol. As agitation increases oxygen transfer also increases and thereby yield.

In the present study, formation of xylitol by *P. stipitis* NCIM 3498 was found to be too negligible at static condition, however xylitol production rose when agitated.

<table>
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<th>Variables</th>
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Table 2: Affect of variables on xylitol production by *P. stipitis* NCIM 3498 using Plackett Burman experimental design.

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at different rpm and attained high concentration (19g/l) at 200 rpm (Fig. 1a). In contrast to our study Bhattacharya et al., (2016) reported maximum xylitol yield of 0.61g/g by *Pichia stipitis* (NCIM 3500) at 150 rpm.

Our results are in agreement with Jin et al., (2005) who investigated fermentation by *P. stipitis* FPL-YS30, under aerobic and oxygen-limited culture conditions and reported xylitol yields of 0.30g/g and 0.27 g/g respectively at aeration rates of 100 and 200rpm.

Our results are similar to the studies of Sampoi et al., (2008) who reported maximum xylitol production with *D. hansenii* at an agitation speed of 200 rpm.

**Effect of temperature on xylitol production**

When optimization of xylitol production is carried out at a temperature range of 25 essaysC-45 essaysC, 30 essaysC was found to be optimum for maximum xylitol production of 22g/l (Fig. 1b). Jeevan et al., (2011) reported 28°C as the optimal temperature for xylitol production by *Pichia* sp from corncob hydrolyzates, which is lesser than 30°C reported in our study.

Similar to our study, Misra et al., (2013) obtained maximum xylitol production of 11.89 g/l at 30°C from corncob hydrolyzate using *C. tropicalis*.

**Effect of media pH on xylitol production**

Optimization of pH range on xylitol fermentation was observed in a range of 4-7. Maximum xylitol production of 25g/l was attained at pH 5 (Fig. 1c). Our reports are in accordance with Mareczky et al., (2015) who reported pH 4.5 as optimum for maximum xylitol production of 21.7 g/L by *H. anomala*.

In contrast to our result, Srivani and Setty, (2012) reported pH 3.5 as optimum for maximum xylitol productivity by *Candida parapsilosis*. 

![Fig. 1: Effect of, agitation speed, temperature and pH on xylitol production.](image)

![Fig. 2: Effect of inoculum percentage and nitrogen source on xylitol production.](image)

![Fig. 3: Effect of co-substrate and xylose concentration on xylitol production.](image)
Effect of inoculum percentage on xylitol production

When inoculum percentage was used in the range of 5-20% v/v for xylitol production, 15% was found to be most favorable for highest xylitol production of 27g/l (Fig. 2a). Our results are supported by observations of Kresnowati et al., (2012) that greater initial cell concentration led to higher xylitol production. However, indifference to our study Bhattacharya et al., (2018) reported 10% (v/v) inoculum of Pichia stipitis (NCIM 3500) as optimum, for formation of maximum xylitol of 32.3 g/L with a yield of 0.63 g/g from concentrated, detoxified water hyacinth hemicellulosic hydrolysate.

Effect of the nitrogen source on xylitol synthesis

Evaluation of the nitrogen source on xylitol production is illustrated in fig. 2b. Ammonium nitrate is shown as best nitrogen source among others for high xylitol accumulation of 29g/l. In contrast to our study Rodrigues et al., (2011) employed Pichia stipitis YS-30 for production of xylitol by providing urea and ammonium sulphate as nitrogen sources and found that xylitol production increased by the addition of urea to the medium.

The xylitol yield obtained in our work is less compared to Mukherji et al., (2013) who reported maximum xylitol yield of 0.852 gm/gm, using cotton seed flour as the organic nitrogen source by novel yeast Pichia Caribbica (HQ222812).

Bhattacharya et al., (2016) reported maximum xylitol production using ammonium sulphate as a nitrogen source. They observed an increase in xylitol concentration and yield from 32.45 g/L to 40.5 g/L and 0.49 g/g to 0.61 g/g respectively with the enhancement of concentration of ammonium sulphate from 1 g/L to 2.5 g/L by P. stipitis (NCIM, 3500).

Effect of co-substrate on xylitol production

The biggest challenge for xylitol production by yeasts is xylose uptake, to regenerate NADPH for xylose reductase activity. Co-substrate utilization has become one of the strategies to overcome this challenge by the continuous provision of NADPH to meet the energy and carbon intermediates required for cell growth and maintenance and thereby improving xylitol production. Glucose is used as the preferred co-substrate over other sugars, due to its low cost and abundant availability (Tamburini et al., 2010; Uppada et al., 2014).

Among the four sugars, arabinose, glucose, maltose and galactose optimized in our study (Fig. 3a) glucose is found as better co-substrate for increased production of xylitol of 31g/L from xylose synthetic media. In contrast to our report, Perez et al., (2016) proposed sucrose to be an ideal co-substrate for maximum xylitol production of 36.11 g/l.

Our result is in accordance with observations of Silva and Felipe, (2006) who obtained an increased yield of xylitol (0.59 g/g) using Candida guilliermondii FTI 20037 when glucose and xylose are used in the ratio of 1:5 which is similar to our study.

Effect of xylose concentration on xylitol production

The influence of xylose on xylitol formation is determined in range of 30g/L to 60 g/L and is depicted in fig. 3b. The xylitol formation has been increased steadily and a maximum xylitol production of 36g/L was obtained at xylose concentration of 60g/L and thereafter gradually decreased. Our results are similar to Ping et al., (2013)
who observed utmost xylitol yield of 0.71 g/g at initial xylose concentration of 60 g/L.

The concentration of xylose 60g/L for maximum xylitol production in our study is less compared to the reports of Kim et al., (2019) who observed highest xylitol production using xylose concentration of 300g/L.

Our results are supported by the findings of Tamburini et al., (2015), who demonstrated that Candida tropicalis DSM 7524 has shown maximum xylitol yield of 71-83%, by consuming xylose between 60 g/L and 80 g/L and further increase of xylose beyond 80 g/L, led to drastic decrease in xylitol yield.

Plackett Burman design

Plackett Burman is orthogonal, fractional two-level factorial design used for identification of important parameters that influence the process of fermentation. This design gives the effect of every variable purely, without confounded interaction with other variables (Naveena et al., 2005).

In the current study, 12 chemical components are screened for their influence on xylitol production by P. stipitis NCIM 3498 using statistical design, Plackett Burman. The experimental design and their responses (xylitol production) are shown in table 2. In the table, each row represents experiments that involve 12 independent variables and each variable with two levels of concentration. The standard T-test was performed using the MINITAB (Release 17, PA, USA). The effect of parameter E, standard error (SE), t-value and probability (P) for the design are represented in table 3. It is observed that the t-test values for xylose, peptone, ammonium nitrate, magnesium sulphate and di-sodium hydrogen phosphate have highly significant values with $> 97\%$ confidence level (Table 3). The analysis also confirms that the above 5 variables played a key role in xylitol production. From a statistical point of view, all other remaining factors do not have any significant effect on xylitol production.

The concentration of xylose 60g/L for maximum xylitol production in our study is less compared to the reports of Kim et al., (2019) who observed highest xylitol production using xylose concentration of 300g/L.

Our results are supported by the findings of Tamburini et al., (2015), who demonstrated that Candida tropicalis DSM 7524 has shown maximum xylitol yield of 71-83%, by consuming xylose between 60 g/L and 80 g/L and further increase of xylose beyond 80 g/L, led to drastic decrease in xylitol yield.

The results of our study are supported by reports of Kumdam et al., (2012) that di-sodium hydrogen phosphate along with $K_2HPO_4$ creates a suitable buffer system in the production medium and hence increase in its concentration lead to increase in xylitol production.

Our study is in accordance to report of Ramesh et al., (2013) who observed peptone, xylose, $MgSO_4\cdot7H_2O$ and yeast extract to be significant variables for xylitol production from comcob hydrolysate using Pachysolen tannophilus (MTTC, 1077) by Plackett Burman model.

Using Plackett Burman design, Hongzhi et al., (2011) reported $(NH_4)_2SO_4, KH_2PO_4$, yeast extract and $MgSO_4\cdot7H_2O$ as significant variables affecting xylitol production.

Pareto Chart

Pareto chart is a graphical representation of Student's t-test. It illustrates different variables affecting xylitol production in significant order (Fig. 4).

Validation

Validation of the model for xylitol production was carried out based on the optimized conditions predicted by Plackett-Burman design. Xylitol production and yield under these optimum conditions were found to be 52g/L and 0.74g/g, respectively (Fig. 5). Xylitol yield in current study 0.74g/g is close to xylitol yield 0.73g/g obtained by Neeru et al., (2013) under optimized fermentative conditions of 32°C, pH 5.7 for 72 h using Pichia stiptis CBS 5773.

Conclusion

Optimization of xylitol production using a statistical model, Plackett Burman was proved to be efficient and can successfully be used for the optimization of components of media to increase xylitol production.

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